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A freshwater mussel species reflects a Miocene stream capture between the Mekong Basin and East Asian rivers

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Abstract

Freshwater mussels belonging to the genus *Cristaria* Schumacher, 1817 (Bivalvia: Unionidae) are widespread from Mongolia to Indochina while the range of one species, *C. plicata* (Leach, 1814), covers two biogeographic subregions, i.e., East Asian (Amur River to Vietnam) and Sundaland (Mekong River basin). We present here a taxonomic revision of the nominal taxon *Anodonta bellua* Morelet, 1866 which was described from the Mekong (Lake Tonle-Sap, Cambodia) but is currently considered a synonym of *C. plicata*. We obtained molecular data for newly collected *Cristaria* representatives from the Mekong's tributaries in Laos, which were found as a divergent species-level phylogenetic clade within the genus that is distant from *C. plicata*. Nevertheless, comparative morphological and morphometric studies did not reveal any significant differences between these two congeners. Our time-calibrated biogeographic modeling reveals that the split between *Cristaria bellua* (Mekong) and *C. clessini* (East Asia) probably occurred in the mid-Miocene (15.8 Ma) and may reflect an ancient stream capture between the Mekong Basin and East Asian rivers.

Key Words

Anodonta bellua, *Cristaria*, East Asia, Laos, Mekong, Miocene, stream capture, Sundaland

Introduction

The freshwater mussel genus *Cristaria* Schumacher, 1817 (Bivalvia: Unionidae) represents a widespread taxon, which commonly occurs in water bodies throughout East Asia and Indochina (Brandt 1974; He and Zhuang 2013; Dang and Ho 2017; Bolotov et al. 2020; Lopes-Lima et al. 2020; Graf and Cummings 2021). According to the current taxonomy, it comprises five species, i.e., *Cristaria plicata* (Leach, 1814), *C. clessini* (Kobelt, 1879),

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C. beirensis Liu & Zhang, 1982, *C. radiata* Simpson, 1900, and *C. truncata* Dang, 1980 (Graf and Cummings 2021). *Cristaria clessini* is endemic to the western parts of Honshu Island, Japan (Lopes-Lima et al. 2020); *C. beirensis* inhabits the Amur Basin, as well as streams draining to the Sea of Japan and the Yellow Sea; the range of *C. radiata* crosses China (Graf and Cummings 2021); and *C. truncata* is distributed in Northern Vietnam (Do et al. 2018). *Cristaria plicata* is considered to be the most widespread and abundant taxon within the genus, the range of which extends throughout the Amur (Russia, northeastern China, and Mongolia) and Yangtze (eastern China) basins, water bodies of central Sakhalin, Japan, South Korea, and northern Vietnam (Prozorova et al. 2004; Klishko et al. 2014, 2016; Do et al. 2018; Wu et al. 2018; Bolotov et al. 2020; Lopes-Lima et al. 2020) as well as being known to occur in the Mekong Basin in Indochina (Haas 1969; Brandt 1974; Nahok et al. 2017; Goncalves et al. 2022). Consequently, the range of *Cristaria*, including *C. plicata*, covers the East Asian and Sundaland subregions, being a unique example of a unionid genus crossing the drainage divide between the Mekong and East Asian rivers (Bolotov et al. 2018, 2020; Goncalves et al. 2022).

Cristaria plicata differs from other species by its large, well recognizable elliptical-rhomboidal shell, which is usually winged posteriorly, as well as by its strong hinge plate with more or less developed lateral teeth (Brandt 1974; He and Zhuang 2013). This species has high economic importance, especially in China, and is actively used for agriculture, the food industry, pearl production, and medical purposes (Patnaik et al. 2016). Under this taxonomic name, a plethora of species was earlier synonymized (Klishko et al. 2014, 2016). In particular, among them there is a nominal taxon, *Anodonta bellua* described by Morelet (1866), from Lake Tonle-Sap, Cambodia ('in lacu Touli-Sap, Cambogensi'). Interestingly, this is the only nominal species of *Cristaria* collected and described from the Mekong Basin. Simpson (1900, 1914) in his comprehensive Unionidae revision regarded *A. bellua* as a valid species belonging to the genus *Cristaria*. This author noted that *C. bellua* is more rhomboid and inflated than *C. plicata* (Simpson, 1914). Preston (1912) in his work also held the same view. Later, Haas (1969) synonymized *C. bellua* with *C. plicata*. Brandt (1974) and He and Zhuang (2013) also listed *C. bellua* as a synonym of the latter species. Brandt (1974) suggested there were two morphotypes of *Cristaria plicata* in the Mekong, such as "a thin-shelled, inflated race from still water pools in the Province of Kon Kaen and a compressed, thicker-shelled race from the Mekong". This author also noticed that "this species has semi-oval glochidia without hooks" and it "cannot be placed among Anodontinae as Haas (1969), Vokes (1967: 213) a.o. suggested" (Brandt 1974; Sayenko et al. 2005; Goncalves et al. 2022). Sayenko et al. (2005) noted that what Brandt named *C. plicata* could be another taxon. He and Zhuang (2013) contradicted Simpson's conclusion about the distinct shell shape of *C. bellua* (see above) and reasoned that specimens of *C. plicata* from Hubei Province, China, also have the same shape characteristics and there are no conchological differences between both species. Dang et al. (1980) recognized *C. bellua* as a valid taxon along with three other *Cristaria* species for the fauna of Vietnam.

In recent studies on Far East Asian and Russian unionids (Bolotov et al. 2020; Lopes-Lima et al. 2020), the molecular and biogeographic information for *Cristaria* species from China, South Korea, Russia, Japan, and Vietnam was summarized. However, DNA sequences for *Cristaria* from the Mekong were not available until now. Interestingly, recent explorations in the Tonle Sap Lake and its tributaries in Cambodia, the type locality of *Anodonta bellua*, did not reveal any *Cristaria* specimens (Ng et al. 2020).

We collected living specimens of *Cristaria* cf. *plicata* from the Mekong Basin during fieldwork in Laos and generated new DNA sequences for subsequent analyses. This work aims to (1) clarify the taxonomy and distribution of *C. plicata*; (2) revise the taxonomic status and estimate the phylogenetic position of the nominal species *Anodonta bellua* Morelet, 1866 from the Mekong Basin; and (3) provide some aspects of the evolutional history of the genus *Cristaria* using paleogeographical, as well as molecular, morphological, and biogeographical information.

Materials and methods

Data sampling

Mussel specimens, preliminarily identified as *Cristaria* cf. *plicata*, were collected from three localities of the Mekong Basin in Laos (Tables 1, 2). Tissue snips for DNA analyses were then preserved in 96% ethanol. The shell and tissue samples were deposited in the Russian Museum of Biodiversity Hotspots (RMBH thereafter), N. Laverov Federal Center for Integrated Arctic Research of the Ural Branch of the Russian Academy of Sciences (Arkhangelsk, Russia).

Table 1. Information on the *COI*, *16S rRNA*, and *28S rRNA* gene sequences of *Cristaria bellua* from the Mekong Basin, Laos.

Specimen Voucher	Locality	NCBI GenBank acc. nos.		
		COI	*16S rRNA*	*28S rRNA*
RMBH biv 813/1	Nam Ngum River	ON704642	ON695881	ON695893
RMBH biv 813/2	Nam Ngum River	ON704643	ON695882	ON695894
RMBH biv 813/3	Nam Ngum River	ON704644	ON695883	ON695895
RMBH biv 853/1	Nam Don River	ON704645	ON695884	ON695896
RMBH biv 853/2	Nam Don River	ON704646	ON695885	ON695897
RMBH biv 891/1	Tributary of Nam Ngiep River	ON704647	ON695886	n/a
RMBH biv 891/2	Tributary of Nam Ngiep River	ON704648	ON695887	ON695898
RMBH biv 891/5	Tributary of Nam Ngiep River	ON704649	ON695888	ON695899

Table 2. Occurrences of *Cristaria* species from Mekong Basin, Indochina based on museum records.

Species	Catalog number	Country	Original locality	Coordinates (Latitude, Longitude)	Collection data
Cristaria bellua	RMBH biv 813	Laos	Nam Ngum River	18.2123, 102.9998	I. N. Bolotov, I. V. Vikhrev, K. Inkhavilay, E. S. Konopleva, Yu. E. Chapurina and locals leg., 26 February 2020
Cristaria bellua	RMBH biv 853	Laos	Nam Don River	17.4781, 104.7630	I. N. Bolotov, I. V. Vikhrev, K. Inkhavilay, E. S. Konopleva, Yu. E. Chapurina and locals leg., 29 February 2020
Cristaria bellua	RMBH biv 891	Laos	Tributary of Nam Ngiep River	18.5783, 103.5998	I. N. Bolotov, I. V. Vikhrev, K. Inkhavilay, E. S. Konopleva, Yu. E. Chapurina and locals leg., 5 March 2020
Cristaria bellua	MCZ_175610	Cambodia	Lake Touli-Sap, Cambodia, Indo China	13.0, 104.0	A. Morelet leg.
Cristaria bellua	BMNH_1965147	Cambodia	Iac Toui-Sap [Cambodia]	13.0, 104.0	A. Morelet leg.
Cristaria bellua	UMMZ_231091	Thailand	River Lam Chi at Gaeng Nam Ton, south of Kon Kaen, [Thailand]	16.3851, 102.7707	23 April 1965
Cristaria bellua	FMNH_296520	Thailand	Thailand, Mun River near Bon Tum	15.3195, 103.6754	R. A. Brandt leg., 5 May 1964
Cristaria bellua	ANSP_162038	Cambodia	Grand Lac, Cambodia	13.0, 104.0	F. Baker leg.
Cristaria bellua	ANSP_56516	Cambodia	Lake Touli-Sap, Cambodia	13.0, 104.0	n/a
Cristaria bellua	MNHN-IM-2022-16006	Thailand	Gaeng Nam Ton, south of Kon Kaen, Thailand	16.385, 102.7707	R. A. Brandt leg., 1974
Cristaria bellua	MNHN-IM-2022-16005	Cambodia	Gr. Lacs, Cambodge	13.0, 104.0	M. Kermorgan leg., 1884
Cristaria bellua	MNHN-IM-2022-16001	Cambodia	Grand Lacs, Cambodge	13.0, 104.0	M. Kermorgan leg., 1884
Cristaria bellua	MNHN-IM-2022-16004	Cambodia	Grand Lak, Cambodia, French Indo-China	13.0, 104.0	F. Baker leg.
Cristaria bellua	MNHN-IM-2022-16003	Cambodia	Ban Don Cau, Mekong	12.4833, 106.0167	Bavay leg.
Cristaria bellua	MNHN-IM-2020-16002	Cambodia	Cambodge, Somron Seng	12.22083, 104.8012	n/a
Cristaria bellua	MCZ_102063	Laos	Houtene, confluence of Nam Hinbourne and Mekong rivers, Laos, French Indo China	17.5851, 104.6098	J. Bequaert leg., 28 February 1934
Cristaria bellua	MCZ_266165	Thailand	Thailand: Gaeng Nam Ton, Nam Kaen, S of Son Kaen	16.3851, 102.7707	R. A. Brandt leg., 4 May 1967
Cristaria bellua	MCZ_280900	Thailand	Thailand: Gaeng Lawa, 12 km NE of Ban Pai, Khon Kaen Prov.	16.1564, 102.6829	R. A. Brandt leg., 4 May 1967
Cristaria bellua	SMF_188910	Thailand	Mekong at Tat Panom	16.9443, 104.7308	R. A. Brandt leg., 19 February 1966
Cristaria bellua	SMF_220858	Thailand	Thailand: Mekong River at Ban Kum, 12 km N of Ban Dan	15.3886, 105.4940	R. A. Brandt leg., 8 May 1967
Cristaria bellua	SMF_220859	Thailand	Mekong River, Ban Dan	15.3239, 105.4930	R. A. Brandt leg., 8 May 1967
Cristaria bellua	SMF_220860	Thailand	Thailand: Gaeng Lawa; Kon Kean, 13 km NE Ban Pai	16.1561, 102.6833	R. A. Brandt leg., 4 May 1967
Cristaria bellua	SMF_283462	Thailand	Thailand: Gaeng Namton; S of Kon Kaen	16.3851, 102.7707	R. A. Brandt leg., 4 May 1967
Cristaria bellua	SMF_319625	Thailand	Lam Chi S Kon Kaen bei Gaen Nam Ton	16.3851, 102.7707	R. A. Brandt leg., 4 May 1967
Cristaria bellua	SMF_319627	Thailand	Songkram River, Wannonivat	17.8655, 103.7741	R. A. Brandt leg., 16 February 1966
Cristaria bellua	SMF_319628	Thailand	Mekong, Tat Panom	16.9443, 104.7308	R. A. Brandt leg., 19 February 1966
Cristaria bellua	SMF_319629	Cambodia	Cambodia: Sekong at Stung Treng	13.5358, 105.9635	R. A. Brandt leg., 26 March 1969
Cristaria bellua	SMF_319630	Laos	Laos: Mekong at Muomng khong	14.1169, 105.8562	R. A. Brandt leg., 26 February 1969
Cristaria bellua	INHS_32578	Cambodia	Tonle Sap Lake, near Porsat (=Pursat or Pouthisat), Cambodia	12.6494, 104.1559	M. Davis leg., 23 January 2004
Cristaria bellua	UF_225991	Cambodia	Kampuchea (Cambodia), Grand Lac	13.0, 104.0	F. Baker leg.
Cristaria bellua	UF_507842	Thailand	Mun River and backwater SSE of Ban Pak Nam	15.2655, 104.9803	J. M. Pfeiffer & L. Page leg., 31 January 2016
Cristaria bellua	Department of Biology, Faculty of Science, Mahasarakham University	Thailand	Tham Ban Sanam Bin Stream, a tributary of the Choen River, Mekong Basin	16.6147, 101.9783	Nahok et al. (2017)

The type specimens, non-type museum lots, and images of *Cristaria* from Laos, Thailand, Cambodia, and East Asia were studied in the Museum of Comparative Zoology (MCZ), Harvard University, Cambridge, USA; Natural History Museum (NHMUK), London, Great Britain; Senckenberg Research Institute and Natural History Museum (SMF), Frankfurt, Germany; Muséum national d'histoire naturelle (MNHN), Paris, France; North Carolina Museum of Natural Sciences (NCSM), Raleigh, USA; Florida Museum of Natural History (UF), Gainesville, USA; Academy of Natural Sciences of Drexel University (ANSP), Philadelphia, USA; Illinois Natural History Survey (INHS), Champaign, USA; Field Museum of Natural History (FMNH), Chicago, USA; and University of Michigan Museum of Zoology (UMMZ), Ann Arbor, USA.

Occurrences of *Cristaria* spp. from the Mekong Basin were obtained based on museum data and published references (Fig. 1, Table 2; Suppl. material 1). The map was made through ESRI ArcGIS 10 software (https://www.esri.com/arcgis). The topographic base of the map was formed using free open sources such as Natural Earth Free Vector and Raster Map Data (https://www.naturalearthdata.com), Global Self-consistent Hierarchical High-resolution Geography, GSHHG v2.3.7 (https://www.soest.hawaii.edu/wessel/gshhg), and HydroSHEDS (https://www.hydrosheds.org).

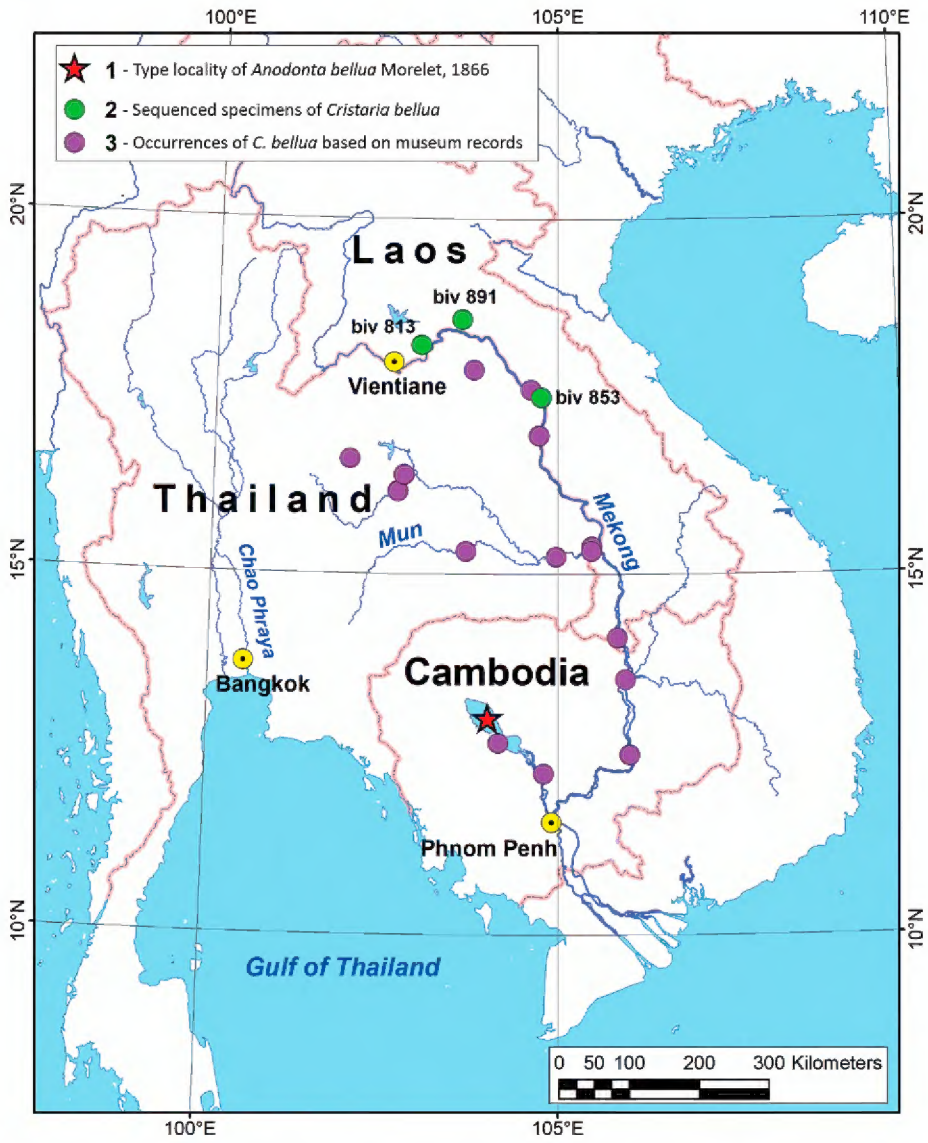

Figure 1. Occurrences of *Cristaria bellua* from the Mekong Basin based on newly collected and museum data. (**1**) type locality of *Anodonta bellua* Morelet, 1866: Lake Tonle Sap, Cambodia; (**2**) sequenced specimens of *Cristaria bellua*: Mekong Basin, Laos; (**3**) occurrences of *Cristaria bellua* based on museum records from Thailand, Cambodia, and Laos: Mekong Basin.

Morphological studies

Morphological analyses were conducted using parameters of shell shape, umbo position, structure of pseudocardinal and lateral teeth, as well as muscle attachment scars (Konopleva et al. 2017, 2019). All specimens were compared with the original descriptions and images of nominal taxa. We analyzed 59 shell contours of the *Cristaria* representatives, including 3 shells of the type specimens of *Anodonta bellua*, 15 shells of newly collected topotypes, 12 shells of *C. plicata* from East Asia, and 29 shells of *C. plicata* from Sundaland (Suppl. material 2). Images of the shells were processed using GIMP v2.10.3 (www.gimp.org). For morphometric analyses, we used Fourier coefficients calculated through SHAPE v1.3 (Iwata and Ukai 2002) as described in previous studies (Konopleva et al. 2017). The results of the Principal Component Analysis (PCA) of Fourier coefficients were visualized using PAST v4.06 (Hammer et al. 2001).

Molecular analyses

Molecular data of mitochondrial (*COI* and *16 rRNA*) and nuclear (*28S rRNA*) markers were obtained for the eight newly collected *Cristaria* specimens (Table 1). Total genomic DNA was extracted from ethanol-preserved tissue samples using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co. KG, Germany), following the manufacturer protocol. Primers for amplification are shown in Table 3. For amplification we applied marker-specific PCR programs as follows: (i) *COI*: 95 °C (4 min), 27 repeats at 95 °C (50 s), 47 °C (50 s), 72 °C (50 s), and 72 °C (5 min); (ii) *28S rRNA*: 95 °C (4 min), 35 repeats at 95 °C (50 s), 57 °C (50 s), 72 °C (50 s), and 72 °C (5 min); and (iii) *16S rRNA*: 95 °C (4 min), 29 repeats at 95 °C (50 s), 46 °C (50 s), 72 °C (50 s), and 72 °C (5 min). Forward and reverse sequence reactions were executed on an ABI PRISM 3730 DNA analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with the ABI PRISM BigDye Terminator v. 3.1 reagents kit. The resulting sequences were checked visually using a sequence alignment editor BioEdit v. 7.2.5 (Hall 1999). The sequences were aligned through the MUSCLE algorithm in MEGA11 (Tamura et al. 2021). Uncorrected genetic p-distances between species within the genus *Cristaria* were calculated through MEGA11 (Tamura et al. 2021).

Phylogenetic and phylogeographic analyses

The mitochondrial phylogeny was based on the *COI* dataset using 130 sequences of *Cristaria* spp., including eight new samples of *Cristaria bellua* from the Mekong (Table 1; Suppl. material 1). Additional sequences of *Cristaria* were obtained from the NCBI's GenBank database (Suppl. material 1). The *COI* dataset was collapsed to 96 unique haplotypes using an online FASTA sequence toolbox (FaBox v1.61; https://birc.au.dk/~palle/php/fabox/; Villesen 2007). The representatives of the subtribe Cristariina (*Beringiana beringiana*, *Buldowskia suifunica*, and *Sinanodonta schrenkii*) and the family Margaritiferidae (*Margaritifera dahurica* and *Gibbosula laosensis*) were used as outgroup. Maximum likelihood phylogenetic analysis was performed using the online server for IQ-TREE v1.6.12 (W-IQ-TREE) with automatic identification of the most appropriate evolutionary models (Chernomor et al. 2016) and ultrafast bootstrapping algorithm (UFBoot) with 5000 replicates (Hoang et al. 2017). Models of sequence evolution for each partition were calculated through Model Finder (Kalyaanamoorthy et al. 2017) based on Bayesian Information Criterion (BIC) as follows: 1st codon of *COI*: F81 + I; 2nd codon of *COI*: TN + G; and 3rd codon of *COI*: TN + I. The Bayesian Inference (BI) phylogenetic analysis was performed in MrBayes v3.2.7 (Ronquist et al. 2012) at the San Diego Supercomputer Center through the CIPRES Science Gateway (Miller et al. 2010). The same evolutionary models were implemented in the *COI* dataset. We used the following parameters: two runs with four Markov chains (three heated and one cold, temperature = 0.2), 15,000,000 generations, and tree sampling every 1000th generation, 15% of trees were discarded as burn-in and

Table 3. Primer sequences using for PCR amplification and sequencing.

Gene fragment	Primer's name	Direction	Sequence (5'-3')	Reference
COI	LCO1490	Forward	ggtcaacaaatcataaagatattgg	Folmer et al. (1994)
	C1-N-2329	Reverse	actgtaaatatatgatgagctca	Simon et al. (1994)
	LoboF1	Forward	kbtchacaaaycayaargayathgg	Lobo et al. (2013)
	LoboR1	Reverse	taaacytcwggrtgwccraaraayca	
16S rRNA	16Sar	Forward	cgcctgtttatcaaaaacat	Palumbi (1996)
	16Sbr	Reverse	ccggtctgaactcagatcacgt	
28S rRNA	C1	Forward	acccgctgaatttaagcat	Jovelin and Justine (2001)
	D2	Reverse	tccgtgtttcaagacgg	

the majority rule consensus tree was calculated from the remaining trees. Convergence of the MCMC chains to a stationary distribution was checked visually based on the plotted posterior estimates using an MCMC trace analysis tool (Tracer v1.7; Rambaut et al. 2018).

For phylogeographic analysis, we trimmed the *COI* sequences of *Cristaria* spp. from the initial length of 659 bp to the final length of 615 bp. A median-joining network was constructed through Network v4.6.1.3 software with default settings (Bandelt et al. 1999). Locality for sequences AS13MT01 and AS13MT02 (Zhang et al. 2013), NC_012716 (Jiang et al. 2010), and KM233451 (Wang et al. 2014) was set as Central East China.

Divergence time estimates and ancestral area reconstruction

A time-calibrated multi-locus phylogeny (3 codons of *COI* + *16S rRNA* + *28S rRNA*) was based on 71 haplotypes of the Unionidae (Suppl. material 1). The best-fit evolutionary models according to BIC were as follows: 1st codon of *COI*: F81+I+G; 2nd codon of *COI*: K3Pu+G; 3rd codon of *COI*: HKY+I+G; *28S rRNA*: TIM2+G; and *16S rRNA*: TIM2+I+G. Instead of using the estimated best-fit models, we used the less complex HKY model with corresponding distributions for each partition to avoid overparameterization (Bolotov et al. 2017). Calculations were performed in BEAST v1.10.4 with a lognormal relaxed clock and Yule speciation process with continuous quantile parametrization as priors (Suchard et al. 2018). We used three fossil calibration points with *Lamprotula*, *Cuneopsis*, and *Cristaria* as MRCA (Bolotov et al. 2017) with an exponential distribution prior: mean (lambda) = 9.3, offset = 34. The MRCA of the Unionidae was set at 152 Ma (Lopes-Lima et al. 2021; Zieritz et al. 2021) with an exponential distribution prior: mean (lambda) = 2.7, offset = 152. Two independent runs of 25,000,000 generations were processed, with sampling every 1000 generations. The resulting tree sets were combined using LogCombiner v1.10.4 with 10% burn-in. The ESS values were checked using Tracer v1.7 (Rambaut et al. 2018) and each value was recorded as >400. A maximum clade credibility tree has been computed with TreeAnnotator v1.10.4.

Ancestral area reconstruction was based on three algorithms, i.e., Statistical Dispersal-Vicariance Analysis (S-DIVA), Dispersal-Extinction Cladogenesis (DEC), and Statistical Dispersal-Extinction Cladogenesis (S-DEC) implemented in RASP v3.2 (Yu et al. 2015) as described in Bolotov et al. (2017). We assigned two possible ancestral areas of the *Cristaria* species: (A) East Asia and (B) Sundaland. The three primary models were combined into an integrative model using the Combine Results option of RASP v3.2 (Yu et al. 2015).

Results

Results of comparative morphological studies and morphometric analyses

The general outlines of newly collected shells of *Cristaria* from Laos are mainly similar to the type specimens of *Anodonta bellua* Morelet, 1866, described from the Mekong Basin (Fig. 2). Comparative morphological analyses did not reveal remarkable differences in the shell shape and teeth structure between representatives of *Cristaria plicata*, the type specimens of *Anodonta bellua*, and newly collected topotypes. Younger individuals usually differ by a well-developed and high post-dorsal wing, which can be smoothed with aging. Old specimens have large, solid, more ovate-elongated shells with more or less developed wings, sharp and long teeth, and well-marked muscle attachment scars.

PCA based on Fourier coefficients revealed six principal components (PCs) (Fig. 3), among which PC1 and PC2 explained the maximum of the total shell shape variance (69.8% and 12.7%, respectively). However, a Kruskal-Wallis test revealed only one significant component PC2 ($P < 0.05$; Suppl. material 3). This component reflects the position of the wing and the equilaterality of shells. Three 95% confidence ellipses corresponding to the topotypes of *Anodonta bellua*, the shells from Mekong Basin, and the shells from East Asia are almost completely overlapped. The lectotype and paralectotypes of *Anodonta bellua* also lie within the 95% confidence ellipses and mainly correspond to the coordinates of topotypes of *Anodonta bellua* and specimens from the Mekong Basin.

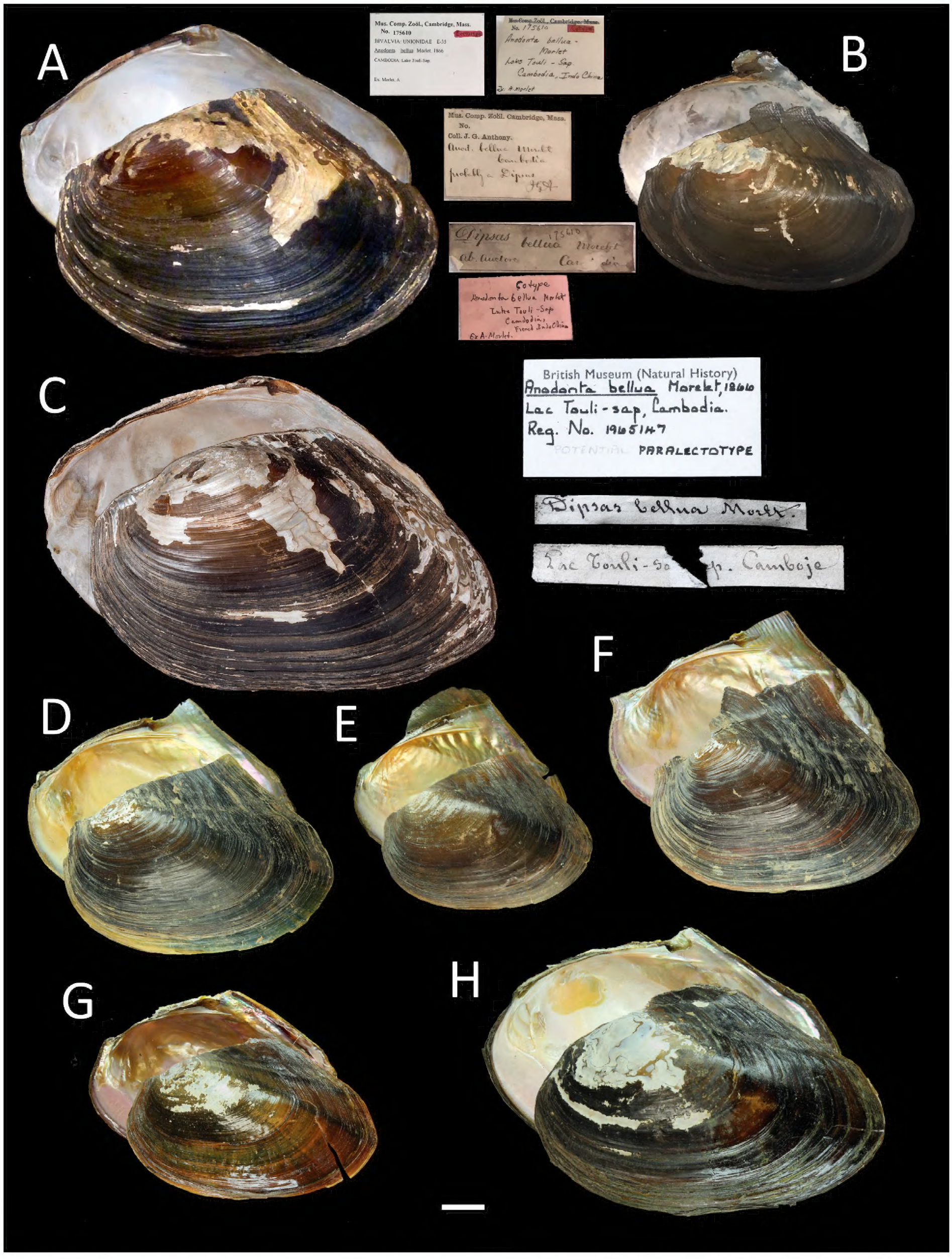

Figure 2. Type specimens and newly collected shells of *Cristaria bellua* (Morelet, 1866) from the Mekong Basin: (**A**) paralectotype MCZ 175610, Lake Tonlé Sap, Cambodia; (**B**) lectotype MCZ 175610, Lake Tonlé Sap, Cambodia; (**C**) paralectotype NHMUK 1965147, Lake Tonlé Sap, Cambodia; (**D**) specimen RMBH biv 813/3, Nam Ngum River, Laos; (**E**) specimen RMBH biv 853/1, Nam Don River, Laos; (**F**) specimen RMBH biv 813/1, Nam Ngum River, Laos; (**G**) specimen RMBH biv 891/1, tributary of Nam Ngiep River, Laos; (**H**) specimen RMBH biv 891/4, the same locality. Scale bar: 2 cm. Photos: Adam J. Baldinger, MCZ [**A, B**]; Kevin Webb, NHMUK [**C**]; and Ekaterina S. Konopleva [**D–H**].

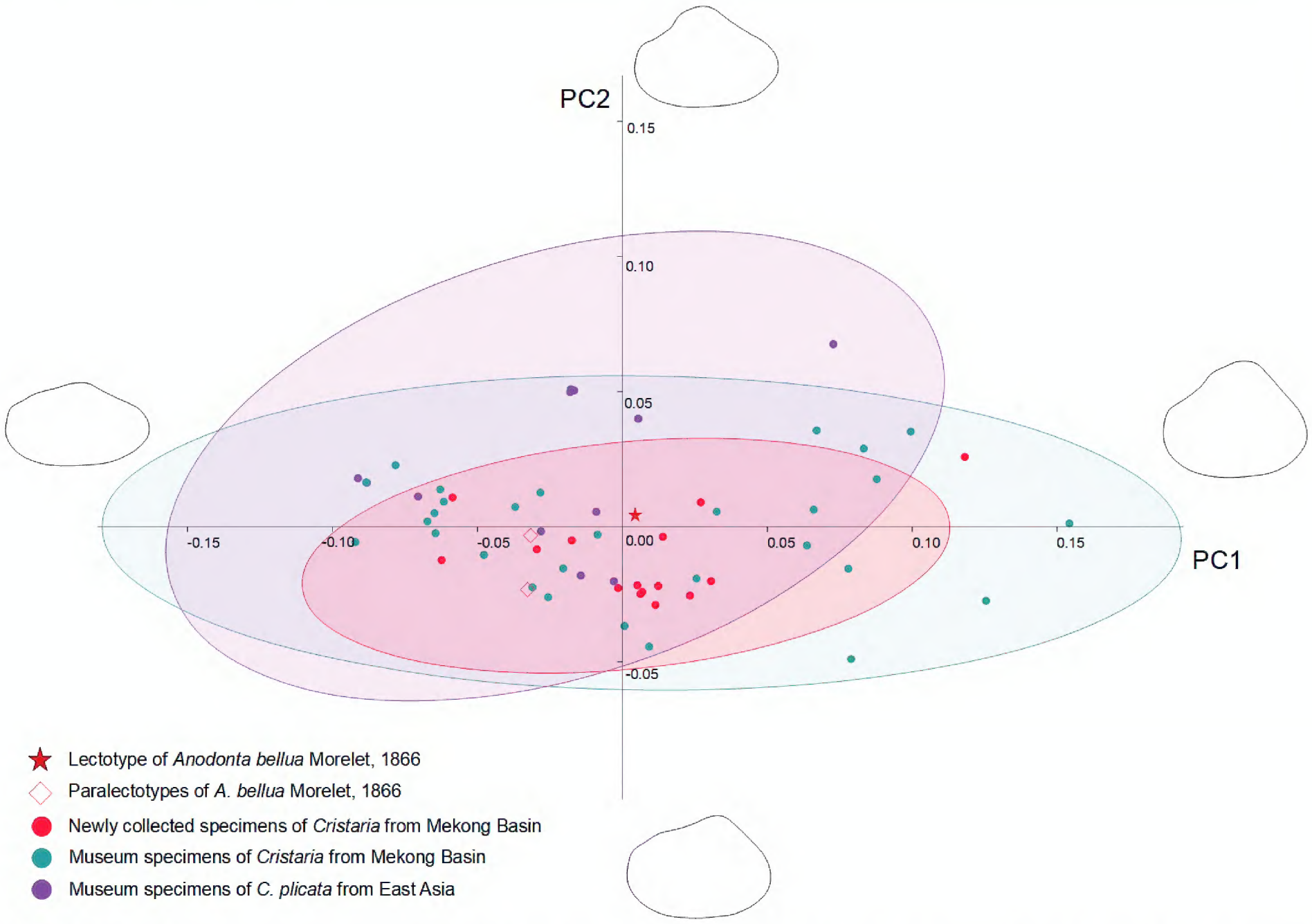

Figure 3. Principal component analysis (PCA) for the first two PC axes obtained using Fourier coefficients of the *Cristaria* shell shapes. The 'extreme' shapes are illustrated by the four synthetic shell outlines. The filled regions show 95% confidence ellipses.

Results of phylogenetic and phylogeographic analyses

The phylogenetic analysis revealed five species-level clades of *Cristaria*, i.e. *Cristaria plicata*, *C. clessini*, *C. truncata*, *C. bellua*, and one undescribed lineage *Cristaria* sp. (Fig. 4). *Cristaria bellua* represents a separate phylogenetic lineage, which is sister to *C. clessini* with an uncorrected *COI* p-distance of 8.0 ± 1.0%. Genetic divergences (mean uncorrected *COI* p-distances, %) calculated between *C. bellua* and other related species of the genus are shown in Table 4. The topologies of the mitochondrial *COI* trees were identical for both the Bayesian and ML analyses. *Cristaria bellua* was recorded as a highly-supported monophyletic clade (1.00/99). Other clades were also well-supported, except for the node leading to *Cristaria bellua* and *C. clessini*, which was moderately supported in the ML analysis (BS = 60).

According to the median-joining network of the *COI* sequences, *C. bellua* also represents a divergent lineage and shares five unique haplotypes (Fig. 5). Two haplotypes from the Nam Ngum River are more divergent from others and differ by five nucleotide substitutions. The *COI* haplotypes of *C. bellua* are more closely related to *C. clessini* from Japan, but do not connect to other *Cristaria* species. The largest *COI* haplotype diversity is observed in *Cristaria plicata*, especially for individuals from China. Three species, *Cristaria truncata*, *Cristaria* sp. and *C. clessini* connect to *Cristaria plicata*.

Fossil-calibrated biogeographic modeling (Fig. 4; Suppl. material 4) suggests that the *Cristaria* MRCA originated within East Asia and Sundaland in the Eocene (mean age = 36.5 Ma, 95% HPD = 34.0–41.2 Ma, BEAST BPP = 1.00) with the probability for an East Asian origin of 76.7% and an East Asia + Sundaland origin of 23.3%. The speciation processes mainly occurred during the Miocene, starting from 20.4 Ma, when the ancestors of two clades (*C. plicata* + *C. truncata* and *C. bellua* + *C. clessini*) diverged.

The split between *Cristaria bellua* and *C. clessini* is placed in the mid-Miocene (mean age 15.8 Ma, 95% HPD = 6.5 – 29.1 Ma), but with a low support value (BEAST BPP

Table 4. Genetic divergences (mean uncorrected p-distances ± standard error estimate, %) between *Cristaria bellua* from Mekong and other related species of the genus *Cristaria* from East Asia based on the mitochondrial *COI* gene sequences.

Species	*Cristaria bellua*	*Cristaria plicata*	*Cristaria truncata*	*Cristaria clessini*
Cristaria plicata	8.7±1.0			
Cristaria truncata	8.5±1.1	4.4±0.7		
Cristaria clessini	8.0±1.0	7.9±1.0	7.6±1.0	
Cristaria sp.	11.8±1.2	10.0±1.1	11.2±1.1	11.4±1.2

= 0.51). This clade most likely originated in East Asia and Sundaland via a vicariance event (probability of 98.4%)

Taxonomy

Family Unionidae Rafinesque, 1820
Subfamily Unioninae Rafinesque, 1820
Tribe Anodontini Rafinesque, 1820
Subtribe Cristariina Lopes-Lima et al. 2017

Genus *Cristaria* Schumacher, 1817

Type species. *Cristaria tuberculata* Schumacher, 1817 (by monotypy).

***Cristaria bellua* (Morelet, 1866)**

= *Anodonta bellua* Morelet, 1866: 167.
= *Margaron* (*Dipsas*) *plicatus* – Lea (1870): 74.
= *Dipsas bellua* – Morelet (1875): 331, Fischer (1891): 222.
= *Cristaria* (s.s.) *bellua* – Simpson (1900): 584.
= *Cristaria* (s.s.) *bellua* – Preston (1912): 281.
= *Cristaria* (s.s.) *bellua* – Simpson (1914): 226.
= *Dipsas plicatus* – Dautzenberg and Fischer (1905): 198.
= *Cristaria* (s.s.) *plicata plicata* – Haas (1969): 387.
= *Cristaria plicata* – Brandt (1974): 278, He and Zhuang (2013): 39, Do et al. (2018): 5; Lopes-Lima et al. (2020): 9.
= *Cristaria bellua* – Dang et al. (1980): 537.

Type and type locality. Lectotype MCZ 175610 (in lacu Touli-Sap, Cambogensi) [Cambodia: Lake Tonlé Sap]; designated by Johnson (1956): p. 107, pl. 1, fig. 1 (Fig. 2B).

Type material. Paralectotype MCZ 175610 (in lacu Touli-Sap, Cambogensi) [Cambodia: Lake Tonlé Sap] (Fig. 2A); Paralectotype NHMUK 1965147 (lac Touli-Sap, Cambodia) [Cambodia: Lake Tonlé Sap] (Johnson 1971: p. 80; Breure et al. 2018) (Fig. 2C).

Material examined. Laos: Nam Ngum River, 18.2123°N, 102.9998°E, Mekong Basin, 26.ii.2020, 3 specimens [RMBH biv 813, all sequenced], Bolotov, Vikhrev, Inkhavilay, Konopleva, Chapurina, and locals leg. (Fig. 2D, F); Nam Don River, 17.4781°N, 104.7630°E, Mekong Basin, 29.ii.2020, 2 specimens [RMBH biv 853, all sequenced], Bolotov, Vikhrev, Inkhavilay, Konopleva, Chapurina, and locals leg. (Fig. 2E); tributary of Nam Ngiep River, 18.5783°N, 103.5998°E, Mekong Basin, 05.iii.2020, 9 specimens [RMBH biv 891, including biv 891/1, biv 891/2 and biv 891/5 sequenced], Bolotov, Vikhrev, Inkhavilay, Konopleva, Chapurina, and locals leg. (Fig. 2G, H). Museum material examined: listed in Table 2.

Re-description. Shell rhomboid, rather large, moderately thin and inflated, sub-solid; high posteriorly, young specimens usually with clearly developed wing or somewhat crest smoothing with aging; ventral margin straight or slightly curved; posterior ridge folded. Umbo small, slightly elevated above hinge line. Periostracum of various coloration, from olive-green to brown and blackish; younger individuals usually with dark-green radial rays running from the umbones to the ventral margin, visible on either side of valve; wider stripes usually located on posterior slope. Nacre whitish, may be tinted with yellow; specimens from the tributary of the Nam Ngiep River differ by a pinkish color, mainly for younger shells. Umbo cavity shallow with a few deep pits. Lateral teeth short, more or less developed on each valve. Pseudocardinal teeth extremely thin or underdeveloped. Anterior muscle scars irregular and usually well-developed, posterior muscle scars more shallow, somewhat crescent-shaped.

Habitat and ecology. The species was recorded from the main channel of the Nam Ngum and Nam Don rivers and a tributary of the Nam Ngiep River, mainly on clay-sandy substrates and at a depth of >1.5–2.0 m. It was found together with representatives of different genera such as *Lens*, *Physunio*, *Hyriopsis*, *Pilsbryoconcha*, *Bineurus*, *Monodontina*, *Scabies*, and *Nyeinchanconcha*. Numerous parasitic mites (Acari: Unionicolidae) and their eggs were discovered on the mantle and gills of *C. bellua*, especially from specimens collected in a tributary of the Nam Ngiep River.

Distribution. Mekong Basin in Laos, Cambodia, and Thailand.

Comments. Morelet (1866) did not state how many specimens were collected and studied for the description of *Anodonta bellua*. In the original description, there was only one set of measurements: shell length = 187 mm; shell height = 123 mm; and shell width = 71 mm (Morelet, 1866). In MCZ, there are two specimens under catalog number 175610. One of them was designated by Johnson (1956) as the lectotype of *A. bellua* and illustrated in his work (Johnson 1956: pl. 1, fig. 1). This author wrote that "this the first time that this species has been figured". Second (larger) specimen was not discussed. Specimen NHMUK 1965147 was considered a paralectotype (Johnson 1971).

Discussion

Morphological and molecular characteristics of *Cristaria bellua*

In the present study, *Cristaria bellua* was distinguished as a distinct species based on molecular and phylogenetic analyses. The *COI* haplotypes of *Cristaria bellua* are relatively close to its congener, *C. clessini*, endemic to Japan (Lopes-Lima et al. 2020). Populations of *Cristaria bellua*, collected from three localities of the Mekong Basin during the field surveys are rather distant, with each sample having its *COI* haplotype (Figs 4, 5), which may reflect their isolated existence in these water bodies. Nevertheless, comparative morphological and morphometric studies did not reveal any significant differences from its other congener, *Cristaria plicata*. Historically, *Cristaria bellua* has been synonymized with *C. plicata* by many authors (Haas 1969; Brandt 1974; He and Zhuang 2013; Do et al. 2018; Lopes-Lima et al. 2020), mainly, due to

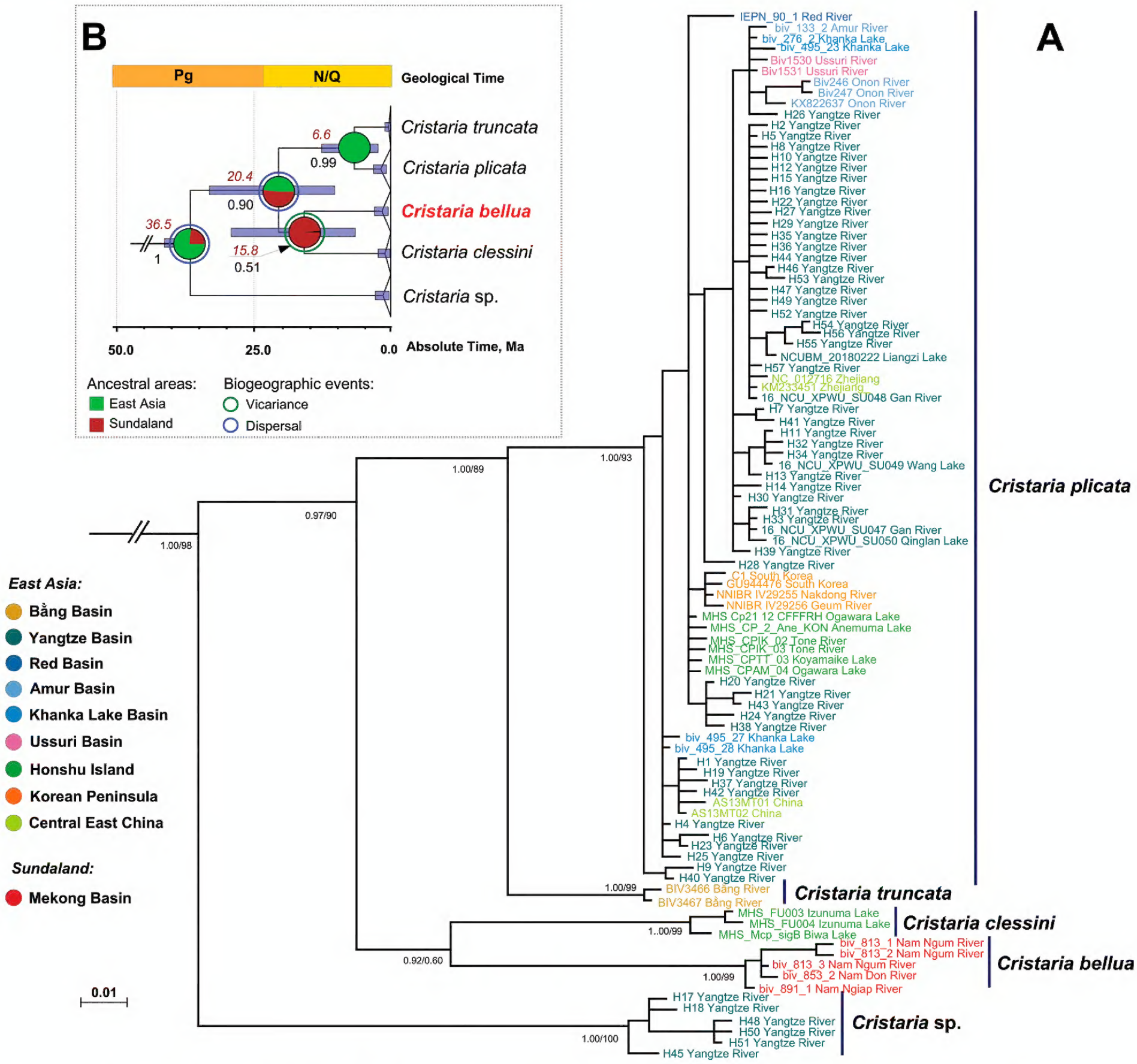

Figure 4. Phylogenetic reconstruction of the genus *Cristaria*. **A.** Bayesian phylogeny of the mitochondrial data set (three codons of *COI*) on *Cristaria* taxa. Scale bar indicates the branch lengths. Black numbers near nodes are Bayesian posterior probabilities (BPP) / ML ultrafast bootstrap support values (BS). The outgroup is not shown. **B.** A fragment of fossil-calibrated Unionidae tree, including the genus *Cristaria*, based on the complete data set of mitochondrial and nuclear gene sequences (five partitions: three codons of *COI+16S rRNA+28S rRNA*) (Suppl. material 4). Black numbers under nodes are Bayesian posterior probabilities (BPP) of BEAST v. 1.10.4; red numbers above nodes are the mean node ages, Ma. Node bars represent the 95% HPDintervals. Stratigraphic chart according to the International Commission on Stratigraphy, 2021 (https://stratigraphy.org/chart).

the high level of conchological similarity of both species. The two species are morphologically very similar and may be distinguished using molecular data and biogeographic patterns only.

Evolutionary biogeography of *Cristaria*

For a long time, it was supposed that the range of *Cristaria plicata* covers the major basins of East and Southeast Asia, including the Mekong River catchment area (Brandt 1974; Lopes-Lima et al. 2020; Graf and Cummings 2021). Such a broad distribution was surprising because this species crosses the drainage divides between separate large freshwater systems such as the Mekong and the East Asian rivers. The crossing of separate drainages was described in previous studies of Southeast Asia, for example, the presence of disjunct mussel species populations in the Mekong and Chao Phraya (Konopleva et al. 2021; Pfeiffer et al. 2021). Earlier, Bolotov et al. (2020) noted that "records from the Mekong Basin may represent a historical human-mediated or natural dispersal event". It is well-known that *C. plicata* has historically been used for food, pearl, nacre, and jewelry production (Landmann et al. 2001; Fiske and Shepherd 2007; Nagai 2013) and was dispersed by man (Schneider et al. 2013). Thereby Schneider et al. (2013) doubted the reliability of the

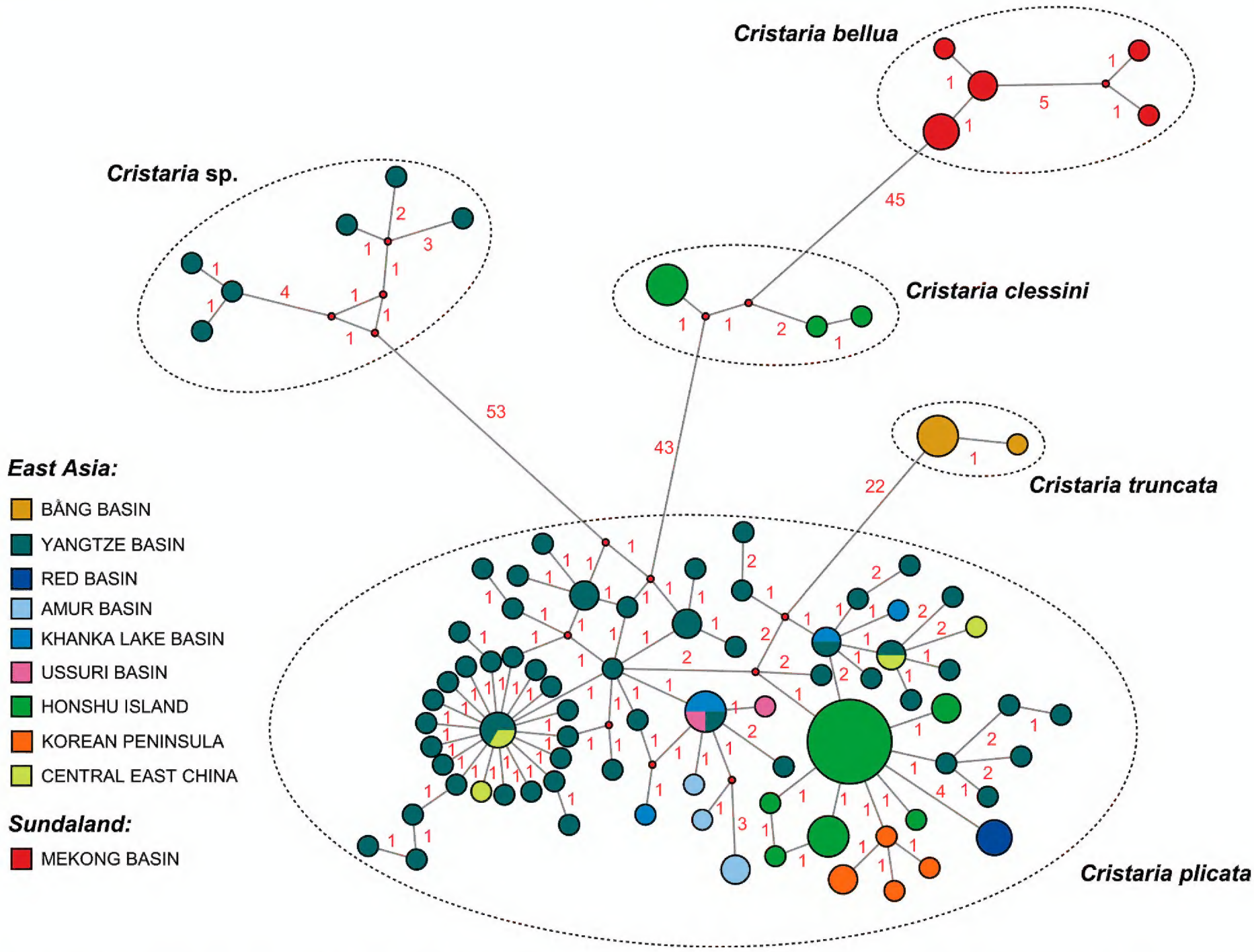

Figure 5. Median-joining network of the *COI* gene sequences of *Cristaria* species ($N = 130$). The red numbers near branches indicate the numbers of nucleotide substitutions between haplotypes. Size of circles corresponds to the number of available sequences for each haplotype (smallest circle = one sequence). Red small circles indicate hypothetic haplotypes.

genus *Cristaria* for paleogeographic reconstructions. In contrast, our molecular analyses showed very high *COI* genetic divergence between *Cristaria bellua* and all its congeners (uncorrected p-distance > 8.0%), and point to long-term isolation in the Mekong Basin with a subsequent speciation event.

Multiple fossils of *Cristaria* were found among the Paleogene Na Duong, Cao Bang, and Rhin Chua mollusk assemblages (Böhme et al. 2013; Schneider et al. 2013). These records were used in our fossil-calibrated phylogenetic modeling as one of the calibration points. According to our model, the diversification of *Cristaria* mainly occurred in the Miocene. The MRCA (Middle Miocene) of *Cristaria bellua* from the Mekong Basin and *C. clessini* from Japan suggests a probable ancient stream capture between the Mekong and the East Asian rivers and the start of the separate evolution of these lineages since this event. The moderate to high level of phylogenetic support for a node of the sister species *Cristaria bellua* and *C. clessini* as well as a high genetic distance between these lineages may indicate the presence of putative additional taxa, which have not yet been sampled, for example from remote and understudied areas of northern Laos, China, Vietnam or the Korean Peninsula.

Several geological studies support the hypothesis of an ancient stream capture between the paleo-Mekong and paleo-Red rivers. According to the geomorphological reconstruction of Clark et al. (2004), in the past, the Upper and Middle Yangtze, Upper Mekong, Upper Salween, and the Tsangpo (Upper Brahmaputra) rivers drained together to the South China Sea through the paleo-Red River. In this work, the Mekong River is considered a major tributary of the paleo-Red River system before the elevation of southeastern Tibet (Clark et al. 2004). Conversely, analyses by Hoang et al. (2009) showed that the Mekong headwaters (as well as the Ayeyarwady and the Salween) have not been connected to the Red River since the Late Miocene. Furthermore, the drainage of the paleo-Red River included the middle part of the present Yangtze and the headwaters of the modern Pearl River (Clift et al. 2008; Hoang et al. 2009).

There are examples of East Asian fish genera, which cross the Mekong drainage divide, with one or several species being endemic to the Upper Mekong. For instance, representatives of *Pareuchiloglanis* species inhabit the Upper and Middle Yangtze, Red, and Pearl River basins and at the same time the Mekong drainage (Li et al. 2020). The same distributional patterns are typical

for another fish genus, *Vanmanenia*, which occurs in the Upper Yangtze, Pearl, and Red River drainages and the Upper Mekong (Lancangjiang) (Li et al. 2019).

Recently, the representative of the typical East Asian fish genus *Carassius* Nilsson, 1832 was described from the Upper Nam Ngum River in the Mekong Basin in central Laos, i.e., *C. praecipuus* Kottelat 2017 (Kottelat 2017). As Kottelat (2017) claimed, the Nam Ngum River together with the Nam Ngiep and Nam Neun have headwaters on the Plain of Jars. One of these rivers, i.e. the Nam Neun, is adjacent to the Ma River Basin and flows through Vietnam into the South China Sea (the Gulf of Tonkin). The fact that the three rivers are separated only by very low divides may suggest a faunal exchange in the past, which has already been shown for a few species (Kottelat 2017). Consequently, *Cristaria bellua* may have been isolated in the Mekong Basin, for example, together with some East Asian fishes.

Further molecular studies and field sampling is necessary to better understand the evolutionary diversification and the biogeography of *Cristaria.*

Distribution and ecology of *Cristaria bellua*

Currently, *Cristaria bellua* is the sole representative of the genus *Cristaria* and the only native member of the subfamily Unioninae in the Mekong River drainage. Live specimens of *Cristaria bellua* were recorded in three water bodies of the Mekong basin in Laos, i.e., the Nam Ngum, Nam Don, and Nam Ngiep rivers. The majority of available museum lots of *Cristaria* from Laos, Cambodia, and Thailand were collected as late as the second part of the 20th century or even earlier (see Table 2). Among the most recent museum records of *Cristaria* (January 2016), only shells from one locality of the Mun River, Thailand were registered (UF 507842). Additionally, one *Cristaria* shell was collected from the Choen River basin in Thailand in 2015 (Nahok et al. 2017). The fact that the present living specimens from Laos were collected from a considerable depth (>1.5–2.0) also revealed that mussels of *Cristaria bellua* are not easily accessible for sampling.

As mentioned in the taxonomic account, specimens of *Cristaria bellua* were infested by parasitic mites (Acari: Unionicolidae). However, this is not surprising, because there are multiple observations of mussel-associated mites and their eggs in the congeneric species *Cristaria plicata* from China (Wen et al. 2006; Wu et al. 2008; Zhang et al. 2018). Probably, *Cristaria bellua* may serve as a nutrition source and shelter for these parasites (Vidrine 1986; Wen et al. 2006).

The Mekong Basin harbors one of the richest fauna of the Unionidae globally with many endemic taxa (Pfeiffer et al. 2018, 2021; Zieritz et al. 2018; Jeratthitikul et al. 2021; Konopleva et al. 2021). The fauna in Laos is threatened by many anthropogenic pressures such as habitat degradation, water pollution, dam construction, and overharvesting of aquatic animals (Bolotov et al. 2014). Hence, *Cristaria bellua* requires conservation attention including accurate monitoring efforts, especially because viable populations in other parts of the Mekong, for instance in the type locality, have not been recorded during recent decades (Ng et al. 2020).

Conclusion

In this study, we revised the taxonomic status and estimate the phylogenetic position of the nominal species *Anodonta bellua* Morelet, 1866, which was earlier considered a synonym of the widespread species *Cristaria plicata* (Leach, 1814). The molecular and phylogenetic analyses support *Cristaria bellua* as a distinct species, which is relatively close to its congener, *C. clessini* (Kobelt, 1879). At the same time, *C. bellua* can hardly be distinguished conchologically from its congener, *Cristaria plicata*.

All known representatives of the genus *Cristaria* are distributed in East Asia, except for *Cristaria bellua,* which inhabits the Mekong River drainage. Phylogeographic modelling revealed that the diversification in the genus *Cristaria* probably occurred in the Miocene. The speciation events in this genus could be linked to the rearrangements of paleo-river basins throughout East and Southeast Asia. We also propose that *Cristaria bellua* may have been isolated in the Mekong Basin together with several East Asian freshwater fish species. Our results highlight that *Cristaria bellua* is a unique representative of the subfamily Unioninae in the Mekong drainage and that this species is of high priority and concern to future conservation efforts.

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Supplementary material 1

List of sequences used in this study, including species names, localities, voucher numbers, and GenBank accession numbers

Authors: Ekaterina S. Konopleva, Ivan N. Bolotov, Ilya V. Vikhrev, Khamla Inkhavilay, Mikhail Yu. Gofarov, Alexander V. Kondakov, Alena A. Tomilova, Yulia E. Chapurina, Tu Van Do, John M. Pfeiffer, Manuel Lopes-Lima, Arthur E. Bogan

Data type: Sequence dataset (Excel file)

Explanation note: List of sequences used in this study, including species names, localities, voucher numbers, and GenBank accession numbers.

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Link: https://doi.org/10.3897/zse.99.90784.suppl1

Supplementary material 2

Freshwater mussel specimens used in geometric morphometric analyses and results of PCA

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Data type: Morphometric dataset (Excel file)
Explanation note: Freshwater mussel specimens used in geometric morphometric analyses and results of PCA
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Supplementary material 3

Results of Kruskal-Wallis test, including Chi-square and P-values for each principal component

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Data type: Morphometric results (Excel file)
Explanation note: Results of Kruskal-Wallis test, including Chi-square and P-values for each principal component.
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Link: https://doi.org/10.3897/zse.99.90784.suppl3

Supplementary material 4

Figure S1. Fossil-calibrated Unionidae tree, including the genus Cristaria, based on the complete data set of mitochondrial and nuclear gene sequences (five partitions: three codons of COI+16S rRNA+28S rRNA)

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Data type: Biogeographic analysis results (Tif file)
Explanation note: **Figure S1.** Fossil-calibrated Unionidae tree, including the genus *Cristaria*, based on the complete data set of mitochondrial and nuclear gene sequences (five partitions: three codons of COI+16S rRNA+28S rRNA). Black numbers under nodes are Bayesian posterior probabilities (BPP) of BEAST v. 1.10.4; red numbers above nodes are the mean node ages, Ma. Node bars represent 95% HPD intervals. Calibration points are marked by red stars. Stratigraphic chart according to the International Commission on Stratigraphy, 2021 (https://stratigraphy.org/chart).
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